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Differential alterations of kainate receptor subunits in inhibitory interneurons in the anterior cingulate cortex in schizophrenia and bipolar disorder

Tsung-Ung W. Woo^{a,b,d}, Kevin Shrestha^a, Christopher Armstrong^a, Martin M. Minns^a, John P. Walsh^a, and Francine M. Benes^{a,b,c,*}

^aProgram in Structural and Molecular Neuroscience, McLean Hospital, 115 Mill Road, Belmont, MA 02478, United States

^bDepartment of Psychiatry, Harvard Medical School, Boston, MA 02115, United States

^cProgram in Neuroscience, Harvard Medical School, Boston, MA 02115, United States

^dDepartment of Psychiatry, Beth Israel Deaconess Medical Center, Boston, MA 02115, United States

Abstract

The aim of this study was to examine whether glutamatergic inputs onto GABA interneurons via the kainate receptor in the anterior cingulate cortex may be altered in schizophrenia and bipolar disorder. Hence, in a cohort of 60 post-mortem human brains from schizophrenia, bipolar disorder, and normal control subjects, we simultaneously labeled the mRNA for the GluR5 or GluR6 subunit of the kainate receptor with [³⁵S] and the mRNA for the 67 kD isoform of the GABA synthesizing enzyme glutamic acid decarboxylase (GAD)₆₇ with digoxigenin using an immunoperoxidase method. The density of the GAD₆₇ mRNA-containing neurons that co-expressed GluR5 mRNA was decreased by 43% and 40% in layer 2 of the anterior cingulate cortex in schizophrenia and bipolar disorder, respectively. In contrast, the density of the GAD₆₇ mRNA-containing cells that expressed GluR6 mRNA was unaltered in either condition. Furthermore, the amount of GluR5 or GluR6 mRNA in the GAD₆₇ mRNA-expressing cells that contained a detectable level of these transcripts was also unchanged. Finally, the density of cells that did not contain GAD₆₇ mRNA, which presumably included all pyramidal neurons, but expressed the mRNA for the GluR5 or GluR6 subunit was not altered. Thus, glutamatergic modulation of inhibitory interneurons, but not pyramidal neurons, via kainate receptors containing the GluR5 subunit appears to be selectively altered in the anterior cingulate cortex in schizophrenia and bipolar disorder.

Keywords

Glutamate; GABA; Gene expression; In situ hybridization; Post-mortem human brain

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*Corresponding author. McLean Hospital, 115 Mill Street, Belmont, MA 02478, United States. Tel.: +1 617 855 2401; fax: +1 617 855 3199. E-mail address: benesf@mclean.harvard.edu (F.M. Benes).

Contributors

Authors T. Woo and F. Benes designed the study, authors M. Minns and J. Walsh conducted the double in situ hybridization experiments, authors K. Shrestha and C. Armstrong conducted the microscopic quantifications, authors K. Shrestha and T. Woo undertook the statistical analysis, and authors T. Woo and F. Benes wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

Conflict of interest

All authors declare that they have no conflict of interest.

1. Introduction

The anterior cingulate cortex (ACCx) specializes in the monitoring, processing, and integration of cognitive and emotive information (Allman et al., 2001; Barbas, 2000; Carter et al., 1998; Devinsky et al., 1995; Paus, 2001) and has long been strongly implicated in the pathophysiology of a variety of neuropsychiatric diseases, among them schizophrenia and bipolar disorder (Benes, 2000; Carter et al., 2001; Chana et al., 2003; Eastwood and Harrison, 2001). Although the precise nature of alteration of neural circuits within the ACCx in these disorders remain to be fully elucidated, several lines of evidence suggest that modulation of information processing by γ -aminobutyric acid (GABA)ergic inhibitory interneurons is compromised. First, the density of cells with a non-pyramidal shape, putative GABA interneurons, has been found to be decreased in both schizophrenia and bipolar disorder (Benes et al., 2000, 2001). Second, the activity of the GABA_A receptor on pyramidal neurons in layers 2 and 3 appears to be markedly upregulated (Benes et al., 1992). Third, the density of GABA interneurons that contain the calcium binding protein calbindin and possibly also those that contain parvalbumin may be decreased in both disorders (Cotter et al., 2002). Fourth, the density of neurons that express the 67 kD isoform of the GABA synthesizing enzyme glutamic acid decarboxylase (GAD)₆₇ appears to be decreased, most prominently in layers 2 and 5, in both schizophrenia and bipolar disorder (Woo et al., 2004). Finally, in the upper cortical layers, GAD₆₅-immunoreactive axon terminal profiles have been observed to be decreased in bipolar disorder, whereas in schizophrenia there appears to be a neuroleptic-related increase in these profiles (Benes et al., 2000).

The activities of GABA interneurons are subjected to feedback and feedforward modulation by glutamatergic inputs from pyramidal cells that are located both locally and in distant cortical or subcortical regions; together, these mechanisms regulate the flow of information within the cerebral cortex by adjusting the spatial and temporal architecture of GABA neurotransmission (Constantinidis et al., 2001, 2002; Pouille and Scanziani, 2001; Rao et al., 1999). Thus, alterations of glutamatergic inputs could contribute to GABA neuronal disturbances in schizophrenia and bipolar disorder. In fact, data from a recent study from our laboratory suggest that, in the ACCx, glutamatergic neurotransmission via the *N*-methyl-D-aspartate (NMDA) class of glutamate receptor expressed by GABA interneurons may be deficient in both schizophrenia and bipolar disorder (Woo et al., 2004). Because glutamate receptors are pharmacologically and functionally very diverse (Cull-Candy et al., 2001), in order to gain a comprehensive understanding of how altered glutamatergic modulation may affect the functional integrity of GABA neural circuits, it would be important to characterize whether other subtypes of glutamate receptors may also be affected.

The kainate receptor family is composed of 5 subunits: the GluR5, GluR6, GluR7, KA1, and KA2 subunits. GluR5, 6, and 7 subunits can form functional homomeric structures, whereas KA1 and KA2 subunits do not form functional homomeric structures but co-assemble with GluR5, 6, and 7 to form heteromeric complexes (Lerma, 2003; Vignes and Collingridge, 1997). It is known that kainate receptors, especially those that contain GluR5 and GluR6, play a key role in mediating neuronal oscillations in the gamma frequency band (Fisahn, 2005). Interestingly, gamma band oscillation deficits have recently been implicated in the pathophysiology of schizophrenia (Brenner et al., 2003; Gallinat et al., 2004; Haig et al., 2000; Hong et al., 2004; Kwon et al., 1999; Light et al., 2006; Spencer et al., 2004; Uhlhaas et al., 2006; Wynn et al., 2005) and bipolar disorder (Bhattacharya, 2001). Furthermore, there seems to be an interesting dissociation in the localization of the GluR5 and GluR6 subunits in that, at least in the hippocampus, GluR5 is abundantly expressed in GABA neurons (Christensen et al., 2004; Cossart et al., 1998; Mulle et al., 2000), whereas GluR6 appears to be more prominently present in pyramidal cells (Bureau et al., 1999; Paternain et al., 2000).

These observations are therefore consistent with the idea that the GluR5 subunit may be more important in mediating inhibitory tone during gamma synchrony whereas the GluR6 subunit may play a more direct role in regulating pyramidal cell excitation (Fisahn, 2005). Based on these observations, in this study, we tested the hypothesis that the expression of the mRNA for GluR5, but not GluR6 in GABA neurons would be altered in schizophrenia and bipolar disorder.

2. Methods

2.1. Subjects

A cohort of 60 human brains from 20 subjects with schizophrenia, 20 subjects with bipolar disorder, and 20 normal control subjects were obtained from the Harvard Brain Tissue Resource Center at McLean Hospital, Belmont, MA (Table 1). The 3 groups of subjects were matched for age, post-mortem interval (PMI), and whenever possible, sex, hemispheric laterality, and pH. The mean freezer storage time (days $\pm$ S.D.) of the brains was not different between the normal control (1407 $\pm$ 555), schizophrenia (1680 $\pm$ 630), and bipolar disorder (1500 $\pm$ 834) groups. The mean pH ($\pm$ S.D.) also did not differ between the 3 groups (Table 1).

Psychiatric diagnoses were established by retrospective review of medical records and an extensive family questionnaire that included the medical, psychiatric, and social history of the subject. The criteria of Feighner et al. (1972) were used to diagnose schizophrenia and the diagnoses of schizoaffective and bipolar disorder were made according to DMS-III-R criteria. Four and five of the subjects with schizophrenia and bipolar disorder, respectively, were not on antipsychotic medications at the time of death (Table 1). The average dosage of antipsychotics that the bipolar subjects (213.3 $\pm$ 282.8 mg) were receiving (expressed as chlorpromazine equivalent dose or CED) was approximately half that of the schizophrenia subjects (412.2 $\pm$ 465.7 mg). None of the subjects were suffering from any substance abuse/dependence disorder at the time of death, as confirmed by a review of urine toxicology reports, medical records, and family questionnaires.

2.2. Tissue preparation

Tissue blocks containing Brodmann's area 24 were removed from fresh brain specimens at the level of the rostrum of the anterior cingulate gyrus between the points at which the gyrus curves above and below the corpus callosum (Benes et al., 2001). The blocks were immediately fixed in 0.1% paraformaldehyde in ice-cold 0.1 M phosphate buffer (pH 7.4) for 90 min, immersed in 30% sucrose in the same buffer overnight, and then frozen in Tissue Tek OCT® (Sakura Finetek, Torrance, CA) on dry ice. Tissue blocks were then sectioned at 10 μ m on a cryostat. Two sections per subject and therefore 6 sections per matched triplet were used. To control for potential variability of hybridization signals between slides, the six sections from each triplet were mounted on 3 slides as follows: 1) normal control + schizophrenia, 2) normal control + bipolar, and 3) schizophrenia + bipolar.

2.3. Double in situ hybridization

2.3.1. Riboprobe preparation

2.3.1.1. Radiolabeled cRNA probe for GluR5 and GluR6 mRNA: The cRNA probes were transcribed in vitro from full-length cDNA clones of the rat GluR5 (Genbank Accession #NM_017241) and GluR6 (Genbank Accession # NM_031508) subunits (kindly provided by Dr. Christine Konradi, McLean Hospital), which are 92% identical to the human sequences. Corresponding sense probes were also generated and it was confirmed that hybridization of sense probes resulted in no specific labeling. Radiolabeled cRNA probe was prepared by first drying down [³⁵S]UTP (500 μ Ci/ml of probe, New England Nuclear, Boston, MA) in a DNA

Speed-Vac (Savant, Farmingdale, NY). 100 ng/μl of the cDNA template, 0.1 M dithiothreitol (DTT), 3 U/μl RNasin, 5 mM NTPs, 0.8 U/μl of T3 or T7 RNA polymerases (for antisense and sense probe, respectively), and 5X transcription buffer were then added. The transcription mixture was subsequently incubated at 37 °C for 1 h. The cDNA template was digested by incubating the mixture with R1Q DNase at 37 °C for 15 min. Unincorporated NTPs were removed by running the mixture through a Stratagene Nuc-Trap (La Jolla, CA) push column. The eluate was collected and probe concentration was determined by scintillation counting. The probes were stored at -20 °C until use.

2.3.1.2. DIG-labeled GAD₆₇ mRNA probe: DIG-UTP-labeled cRNA probes were transcribed using 100 ng of linearized cDNA subclones spanning nucleotides 1053-1723 (Genbank Accession #NM_000817) within the coding region of the human GAD₆₇ gene in the presence of 0.1 M DTT, 3 U/μl RNasin, 0.8 U/μl of T3 and T7 RNA polymerases, 10 mM of ATP, CTP, and GTP, 6.5 mM of UTP, and 3.5 mM of DIG-labeled UTP (Boehringer Mannheim, Indianapolis, IN). The mixture was incubated at 37 °C for 1 h. cDNA template was digested with RQ1 DNase. The corresponding sense probe was also generated and hybridization of sense probe produced no hybridization signals that were above background. Probe concentration was determined using a standard with known concentrations.

2.3.2. Hybridization—To ensure adequate tissue penetration, the GluR5 and GluR6 probes were hydrolyzed into fragments of about 800 base pairs with an equal volume of sodium bicarbonate/carbonate buffer (pH 10.2; 40 mM NaHCO₃, 60mM Na₂CO₃) at 60 °C for 6-10 min. The reaction was stopped by adding 0.08 vols. of 2 M sodium acetate in 6.25% glacial acetic acid. Probes were reconstituted in a hybridization buffer consisting of 50% formamide, 0.1% yeast tRNA, 10% dextran sulfate, 1X Dehardt's solution, 0.5 M ethylenediamine tetraacetic acid (EDTA), 0.02% sodium dodecyl sulfate (SDS), 4X saline-sodium citrate (SSC) buffer, 10 mM DTT, and 0.1% ssDNA, at a final concentration of 0.4 ng probe/μl hybridization buffer. Before hybridization, mounted tissue sections were air dried and warmed to room temperature. They were then post-fixed in 4% paraformaldehyde for 10 min and incubated in 0.1 M tetraethylammonium (TEA) for 5 min at room temperature before being dehydrated in a graded series of ethanol. Probes were then added to slides for hybridization in a pre-warmed, humidified dish. Sections were covered with coverslips and incubated at 64 °C for 3 h. At the end of hybridization, coverslips were soaked off in 4X SSC in the presence of 100 μl of βMer. Tissue was then incubated in 0.5 M NaCl/0.05 M PB for 10 min, 0.5 M NaCl with 0.025 mg/ml RNaseA at 37 °C for 30 min, followed by a high stringency wash with a solution containing 50% formamide, 0.5 M NaCl/0.05 M PB, and 100 μl βMer at 63 °C for 30 min. Sections were finally washed overnight in 0.5X SSC with 20 mM βMer ETOH at room temperature.

2.3.3. Visualization of DIG labeling—After incubation in blocking buffer (100 mM Tris-HCL, 150 mM NaCl pH 7.5, the sections were placed in buffer containing 3% normal goat serum (NGS) + 0.3% Triton X100) and a 1:200 dilution of sheep anti-DIG antibody conjugated with peroxidase enzyme (Roche Diagnostics, Indianapolis, IN) and were incubated overnight at 4 °C. The sections were washed in buffer and a peroxidase reaction product was localized using a Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, CA) and diaminobenzidine.

2.3.4. Emulsion autoradiography—The slides were apposed to X-ray film (Kodak Biomax MS) for 6 days for determination of whether sufficient autoradiographic signal was present. The slides were then dipped in emulsion (Kodak NTB-2), air dried, and stored at 4 °C in the dark for 4 weeks. After development Kodak D-19 developer, the slides were counterstained with methyl green and coverslipped.

2.3.5. Quantification of GAD₆₇, GluR5, and GluR6 mRNA expression—All microscopic analyses were conducted under strictly blind condition. Quantification for the GluR5/ GAD₆₇ and GluR6/GAD₆₇ experiments was performed by K. S. and C. A., respectively. [³⁵S]-labeling of GluR5 or GluR6 mRNA appeared as clusters of silver grains after processing for emulsion autoradiography (Fig. 1). DIG labeling, in the form of a brown DAB reaction product, was visualized under a brightfield microscope equipped with polarizing filters to enhance the optical density of the reaction product. Neurons that were single-labeled with DIG or [³⁵S] and those that were double-labeled with DIG + [³⁵S] (Fig. 2) were identified on images captured on a computer screen using a Leica Laborlux microscope, which was equipped with a solid state CCD video camera connected to a Bioquant Nova Image Analysis System (R&M Biometrics, Memphis, TN). Using a 100× oil immersion objective lens at a final magnification of 1000×, the distribution of both single- and double-labeled neurons within a 250 μm-wide column extending from the pial surface to the border between layer 6 and the subcortical white matter in Brodmann's area 24 were obtained for each section. Neighboring sections were stained with cresyl violet for the identification of Brodmann's area 24 based on well-defined cytoarchitectonic criteria, as previously described (Benes et al., 1986), and for the determination of laminar boundaries. Densities of single- and double-labeled neurons for each cortical layer were then obtained by dividing cell counts by laminar areas. For silver grain clusters, those with grain density that was at least 2X above background (see below) were defined as specific labeling. Intra-rater reliability, as assessed by counting and recounting profiles within the same column, was established to be 93-97% before the actual data collection process was begun. The actual data collection process for each experiment was completed in 3 months.

To quantify the expression level of mRNA for the GluR5 and GluR6 subunit in individual GAD₆₇ mRNA-positive (+) or GAD₆₇ mRNA-negative (-) cells, the area occupied by each grain cluster was outlined using a cursor displayed on the computer monitor. For each cluster, this was performed according to the principle of including the largest number of grains within the smallest possible area. The cluster area was measured by highlighting the grains with a thresholding subroutine. This threshold was held constant and the light intensity was adjusted to ensure that the size of the grains was neither under-nor over-saturated. This procedure was consistently followed throughout the entire study. The area covered by autoradiographic grains within the cluster area was automatically computed based on the threshold value and was represented as a pixel count for GluR5 or GluR6 transcript expression level. The pixel count was expressed as a function of cluster area. By subtracting the background grain density (i.e. pixel count of the area covered by autoradiographic grains per unit area in μm² in the white matter), the corrected GluR5 or GluR6 expression level was obtained. For each diagnosis group, a histogram of the distribution of grain density was plotted. The average GluR5 or GluR6 expression level in GABA interneurons (i.e. cells positive for GAD₆₇ mRNA) for each cortical layer for each case was then computed.

2.4. Statistical analyses

The densities of single-(GAD₆₇+, GluR5+, or GluR6+) and double-(GAD₆₇+/GluR5+or GAD₆₇+/GluR6+) la-beled neurons were compared between subject groups across layers 1 through 6 using repeated-measures analysis of variance (ANOVA), with diagnosis as the between-groups factor, layer as the within-group factor, and repeated-measures on layer. For post-hoc analyses, two-tailed unpaired *t* tests were used. To detect any differences in the expression of the mRNA for the GluR5 or GluR6 subunit in individual GAD₆₇ neurons between the 3 subject groups, the non-parametric Kruskal-Wallis test was used, as frequency histograms revealed that the grain density data were not normally distributed. In order to evaluate the potential effects of confounding variables, such as age, sex, PMI, brain pH, freezer storage time, and exposure to antipsychotic medications and the mood stabilizer divalproex,

simple Pearson correlations were obtained for the individual groups and when the control group was combined with the schizophrenia and bipolar disorder group, respectively. Although some of the bipolar subjects were receiving other mood stabilizers such as lithium, carbamazepine, etc. at the time of death, statistical evaluation was not possible due to small sample sizes. In addition, for each density measure, an analysis of covariance (ANCOVA) was performed to understand how these confounding variables might have affected our results. Because none of the conclusions derived from our findings were affected by the ANCOVAs, only results from repeated-measures ANOVAs are reported. Potential effects of nominal variables such as hemispheric laterality and sex on our findings were evaluated by using two-tailed unpaired *t* tests (for cell density measures) and Mann-Whiney tests (for silver grain density data) to compare the data from the two hemispheres and those from the two sexes, both within individual groups and when cases from the control group were combined with those from the schizophrenia and bipolar group, respectively.

3. Results

3.1. Density of all GAD67 mRNA-expressing neurons

The repeated-measures ANOVA model revealed a significant diagnosis effect ($F_{2,57} = 5.87$; $p = 0.0048$). Furthermore, the density of GAD₆₇ mRNA-expressing neurons exhibited the most prominent change in layer 2 (Fig. 3), with a 42% and 31% reduction in subjects with schizophrenia ($t = 4.69$; $p = 0.0001$) and bipolar disorder ($t = 3.42$; $p = 0.001$), respectively, when compared to the normal control subjects. Besides layer 2, the density of these neurons was also significantly decreased in layer 5 in the schizophrenia subjects by 24% ($t = 2.44$; $p = 0.019$). This pattern and magnitude of reduction in the GAD₆₇ mRNA-expressing neurons are virtually identical to what was observed in a previous study (Woo et al., 2004).

3.2. Density of GAD₆₇ mRNA-containing neurons that expressed GluR5 mRNA

The effect of diagnosis was significant ($F_{2,57} = 5.91$; $p = 0.0046$). Thus, in the subjects with schizophrenia, density of the double-labeled neurons was significantly decreased by 43% ($t = 3.29$; $p = 0.002$) in layer 2 (Fig. 4). The density of these neurons also appeared to be decreased in layer 5 by about 33% ($t = 2.39$; $p = 0.022$). In the bipolar subjects, the density of the double-labeled was significantly decreased by 40% in layer 2 ($t = 3.01$; $p = 0.005$), whereas the density of these neurons in other cortical layers was unchanged.

3.3. Density of GAD₆₇ mRNA-containing neurons that expressed GluR6 mRNA

In contrast to the GABA cells that expressed the mRNA for the GluR5 subunit, the density of the GluR6 mRNA-expressing GABA cells was not altered in either schizophrenia or bipolar disorder ($F_{2,57} = 0.41$; $p = 0.67$, Fig. 4).

3.4. Density of GAD₆₇ mRNA-containing neurons that did not express GluR5 mRNA

The GABA cells that did not express GluR5 mRNA also appeared to be affected in both schizophrenia and bipolar disorder (Fig. 5); the effect of diagnosis on the density of these neurons was statistically significant ($F_{2,57} = 3.50$; $p = 0.037$). This reflected the 43% ($t = 3.39$; $p = 0.002$) and 39% ($t = 3.01$; $p = 0.005$) reductions in neuronal density in layer 2 in subjects with schizophrenia and bipolar disorder, respectively.

3.5. Density of GluR5 mRNA-containing neurons that did not express GAD67 mRNA

In contrast to the GABA cells that expressed GluR5 mRNA, expression of GluR5 mRNA in cells that did not contain GAD₆₇ mRNA, which presumably included all pyramidal neurons, was not altered in schizophrenia or bipolar disorder ($F_{2,57} = 0.158$; $p = 0.85$; Fig. 6).

3.6. Expression level of GluR5 and GluR6 mRNA

The frequency histograms of GluR5 or GluR6 mRNA expression level per GAD₆₇⁺ cell suggest that there appears to have no significant diagnosis effect on grain densities (Fig. 7). This was confirmed by a Kruskal-Wallis test. Furthermore, the amount of GluR5 or GluR6 mRNA expression in non-GAD₆₇-expressing cells, which presumably included all pyramidal cells, was also unchanged in either disorder (data not shown). Finally, the average size of the silver grain clusters on any of the single-or double-labeled cells did not differ between the 3 groups (Table 2).

3.7. Potential confounding variables

We examined the potential confounding variables, such as age, PMI, brain pH, hemispheric laterality, and medication exposure, on our findings. None of these factors appear to have influenced our results. Among these variables, pH was perhaps particularly important because it is an indirect indicator of the quality and integrity of mRNA (Eastwood et al., 1997; Harrison et al., 1995; Kingsbury et al., 1995). Of note, this measure was not significantly different among the 3 subject groups (Table 1). Furthermore, in our statistical analyses, we found no correlation between pH and any of our density measures either in individual diagnostic groups or when subjects from the disease groups and those from the normal control group were combined. ANCOVAs incorporating pH as a covariate also did not significantly alter the effect of diagnosis on the cell density measures. Similar analyses with CED revealed that exposure to antipsychotic medications was not significantly correlated with any of our dependent measures, nor did it contribute to the observed differences in the neuronal density measures between diagnostic groups. Furthermore, the densities of GAD₆₇⁺/GluR5⁺ neurons in 3 (cases 28, 31, and 39) of the 4 subjects with schizophrenia who were neuroleptic-free were in fact lower than the average density in the neuroleptic-treated subjects by almost 2-fold, consistent with the idea that alterations of these neurons may reflect the underlying disease process of schizophrenia. In fact, some of the beneficial effects of antipsychotic medications, including haloperidol and clozapine, could potentially be mediated in part by their capacity to increase kainate receptor binding (Schmitt et al., 2003).

Within the bipolar group, 8 subjects were on divalproex at the time of death; we found no evidence of any correlation between the dosages of divalproex and any of the density measures. Statistical evaluation of the potential effects of other medications, such as other classes of mood stabilizers, on our results was not feasible due to the relatively small numbers of subjects who were on these medications. However, for this very same reason, it is unlikely that these medications would have significantly biased our results.

4. Discussion

4.1. Summary of findings

We have demonstrated that the density of GAD₆₇ mRNA-expressing neurons appears to be markedly decreased in layer 2 and, to a lesser extent, in layer 5 of the ACCx in schizophrenia whereas in bipolar disorder, the reduction in the density of these cells seems to be confined to layer 2. These findings are strikingly consistent with our previous observations (Woo et al., 2004) in a largely different cohort of subjects (see Table 1) and are in line with an increasing number of studies establishing the fact that disturbances of GABA interneurons in the cerebral cortex appear to represent a core pathophysiologic feature of these disorders (Benes and Berretta, 2001; Costa et al., 2004; Lewis et al., 2005). A challenge is to further delineate how the pre- and post-synaptic elements of GABA interneurons may be altered. Toward this end, in support of earlier theoretical work (Olney and Farber, 1995), recent data from our laboratory suggest that glutamatergic neurotransmission on GABA interneurons via the NMDA receptor may be deficient in schizophrenia and bipolar disorder (Woo et al., 2004). Here, we extend

this previous finding to illustrate that, besides the NMDA receptor, the GluR5-containing kainate class of glutamate receptors may also be involved in the deficits of glutamatergic modulation of GABA neurotransmission. At this point, we do not know whether changes in glutamate receptor expression in GABA interneurons also occur at the protein level, but our finding is consistent with a recent study showing that the density of cells that express kainite receptors, as revealed immunohistochemically using an anti-GluR5/6/7 antibody, appears to be decreased in the orbitofrontal cortex in schizophrenia (Garey et al., 2006). However, two studies using Western blotting failed to detect any changes in the expression of GluR5 protein in homogenized ACCx in schizophrenia (Breese et al., 1995; Breese and Leonard, 1993). Likewise, Zavitsanou et al. also failed to identify any changes in kainite receptor binding in the ACCx in subjects with schizophrenia (Zavitsanou et al., 2002). Nevertheless, these methodologies may not have the sensitivity to detect changes at the cellular level in the magnitudes that were seen in the present study.

4.2. Methodologic considerations

In situ hybridization labeling with [^{35}S] is more sensitive (by approximately 10%) in the detection of changes in transcript expression than non-radioactive markers, such as DIG (Stone et al., 1999). In the present study, because DIG was used to label the GAD₆₇ transcript, it is possible that we may have underestimated the true numerical density of GABA interneurons. However, this potential confound is clearly insufficient to account for the 24-53% and 31-35% reduction in DIG-labeled GABA cells in schizophrenia and bipolar disorder, respectively, that was observed in this and in a previous study (Woo et al., 2004). Previously, it has been demonstrated that, in schizophrenia, the density of Nissl-stained, non-pyramidal, presumably GABA interneurons in the ACCx may be decreased by 12-15% (Benes et al., 1986, 1991, 2001; Todtenkopf et al., 2005). These observations are consistent with the idea that in the ACCx cell loss may occur in schizophrenia. However, even if 12-15% of GABA cells are lost, it is insufficient to account for the magnitude of the observed reduction in GAD₆₇ mRNA-expressing neurons. Thus, it appears that in many of the GABA interneurons the expression of the GAD₆₇ transcript is reduced to a level that is no longer experimentally detectable. In contrast, in bipolar disorder, GABA interneurons have been found in previous cell counting studies to be reduced by 27-35% (Benes et al., 1986, 1991, 2001; Todtenkopf et al., 2005). This magnitude of reduction is numerically similar to the reduction in GAD₆₇ mRNA-expressing cells observed in this and in the previous study (Woo et al., 2005), raising the possibility that these cells may be lost in bipolar disorder.

Another issue that should be addressed is our data collection procedure. For instance, in the quantification of GAD₆₇⁺/GluR5⁺ or GAD₆₇⁺/GluR6⁺ cells, we included only those with silver grain density that was 2X above background, because the exclusive majority (N90%) of the double-labeled cells that were counted met this criterion. In other words, we assume that grain density that was less than 2X that of background resulted from non-specific labeling. However, because this is an arbitrary cut-off, it is possible that among the 10% of the cells that were excluded, some of them may in fact have been GABA cells that expressed a very low level of NR2A mRNA. In the case of the GAD₆₇⁺/GluR5⁺ cells, the possible exclusion of this relatively small contingent of cells should not have affected our conclusions, given the magnitude of change in the density of these cells was observed to be in the 40% range. In the case of the GAD₆₇⁺/GluR6⁺ cells, we cannot rule out the possibility that a very small number of GluR6-expressing GABA cells (i.e. <10%) may in fact be altered but was not detected in this study. Conversely, we also cannot exclude the possibility that, for some of the cells with grain density greater than 2X that of background, the labeling might actually represent a non-specific event. In this case, we may have underestimated the magnitude of reduction in GluR5-expressing GABA cell density, but, again, our conclusions would not have been affected.

4.3. Pathophysiologic implications

The reduction in the density of GluR5 mRNA-expressing GABA cells may reflect either a loss of these neurons or reduced expression of mRNA such that the amount of GluR5 mRNA in a subset of GABA interneurons may be decreased to an experimentally undetectable level. However, these two scenarios need not be mutually exclusive. In the present study, our data suggest that, in the human ACCx, approximately 25% of all GABA neurons express a detectable level of GluR5 mRNA. Furthermore, in layer 2 of the ACCx, the density of the GluR5 mRNA-expressing GABA neurons appears to be decreased by about 40%, which amounts to roughly 10% of the entire GABA cell population, in both schizophrenia and bipolar disorder. Interestingly, data from previous cell counting studies, as discussed above, indicate that the density of GABA interneurons in layer 2 of the ACCx is decreased by 12-15% in schizophrenia (Benes et al., 1986, 1991, 2001; Todtenkopf et al., 2005). Thus, it is possible that, in schizophrenia, the GABA cells that express GluR5 may in fact be lost. However, in bipolar disorder, because the reduction in the density of GABA cells was in the 27-35% range (Benes et al., 1986, 1991, 2001; Todtenkopf et al., 2005), if GABA cell loss does occur, those that do not express GluR5 must also be affected.

Our observation of a reduction in the density of GAD₆₇⁺/GluR5⁺ neurons is consistent with a recent study in which it was found that, using film autoradiography, the amount of mRNA for the GluR5 subunit was reduced in the prefrontal cortex in schizophrenia, whereas the amount of mRNA for the other kainate receptor subunits was not altered (Scarr et al., 2005). Moreover, our study extends this finding by demonstrating that the reduction in the expression of GluR5 mRNA occurs preferentially in the GABA interneurons, whereas pyramidal cells may not be affected.

Hypofunction of NMDA receptors on GABA interneurons may play a central role in the pathophysiology of schizophrenia (Coyle, 2004; Cunningham et al., 2006; Deutsch et al., 2001; Kinney et al., 2006; Olney and Farber, 1995; Olney et al., 1999; Woo et al., 2004). As a result of diminished tonic inhibition, pyramidal neurons that are immediately downstream to these GABA cells may become hyperactive. Consequently, the GABA cells that receive inputs from these hyperactive pyramidal neurons via either feedback or feedforward projections may then be rendered susceptible to excitotoxic injury (Benes et al., 1996; Deutsch et al., 2001; Olney and Farber, 1995; Olney et al., 1999). Thus, the reduction in the expression of the mRNA for GluR5 in at least some of the GABA neurons may, in part, represent a compensatory response to the excessive glutamatergic stimulation they receive. When downregulation of glutamate receptors, together with other compensatory machinery, remains insufficient to mitigate the excitotoxic effects of hyperglutamatergic inputs, cell death could result. Consistent with this line of reasoning, it is of interest that in a recent microarray study, 19 of the 44 genes that are associated with apoptosis were found to be markedly upregulated in the hippocampus in bipolar disorder (Benes et al., 2006). In contrast, in schizophrenia, a number of genes that are linked to the apoptosis cascade were in fact found to be downregulated, which, among other possible interpretations, may reflect the successful compensation for the hyperglutamatergic state in schizophrenia. These two latter observations also underscore an important principle that despite the fact that the observed alterations in GABA neural circuits may appear to be similar between schizophrenia and bipolar disorder, the underlying molecular cascades associated with these alterations could be quite different.

Because subpopulations of GABA interneurons regulate different aspects of neural circuitry functions (Cauli et al., 1997; Gupta et al., 2000; Markram et al., 2004; Wang et al., 2004), an important issue that needs be addressed is the identity of the GABA cells in which GluR5 expression is reduced. It is of interest that the GluR6 subunit, in addition to its localization in the somatodendritic domain of GABA cells, appears to be also present on the presynaptic terminals of the GABA cells that target other GABA interneurons (Mulle et al., 2000).

Therefore, based on our finding that the GluR6-containing GABA cells seem to be unaltered in schizophrenia or bipolar disorder, it can be hypothesized that the functional integrity of the interneuron-targeting GABA cells, many of which contain the calcium-binding protein calretinin (CR), may be relatively preserved in these conditions. This hypothesis is in fact consistent with findings of several previous studies demonstrating that the CR-containing neurons seem to be unaltered in these disorders, but other classes of GABA neurons, such as those that contain the calcium-binding protein parvalbumin (PV), which target the somata and axon initial segments of pyramidal cells, or calbindin, which target pyramidal cell dendrites, may be preferentially involved (Beasley et al., 2002; Cotter et al., 2002; Daviss and Lewis, 1995; Hashimoto et al., 2003; Reynolds et al., 2001; Woo et al., 1998). It is of interest that GluR5 subunit activation has been shown to massively increase tonic inhibition of the somata and apical dendrites of pyramidal neurons (Cossart et al., 1998), raising the possibility that these subunits may be prominently expressed by the PV-containing neurons. Because PV-containing neurons play a key role in synchronizing neural oscillations in the gamma frequency band (Cunningham et al., 2006), the generation of gamma oscillation as a result of kainate receptor stimulation (Fisahn, 2005) may be mediated by these neurons. Taken together, these data point to the possibility that the observed reduced expression of GluR5 subunit may contribute to the pathophysiology of schizophrenia and bipolar disorder by disturbing gamma band synchrony (Bhattacharya, 2001; Brenner et al., 2003; Gallinat et al., 2004; Haig et al., 2000; Hong et al., 2004; Kwon et al., 1999; Light et al., 2006; Spencer et al., 2004; Uhlhaas et al., 2006; Wynn et al., 2005).

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Fig. 1. Darkfield photomicrographs showing the distribution of neurons that express the GluR5 or GluR6 mRNA in the human ACCx. In general, the density of GluR6 mRNA-expressing neurons tends to be higher in all layers than that of the GluR5 mRNA-containing neurons. Also note that the density of GluR5 mRNA-expressing neurons appears to be slightly decreased, especially in layer 2, in both schizophrenia and bipolar disorder whereas the distribution of the GluR6 mRNA-expressing neurons appears to be unaltered in either disorder. Scale bar = 50 μ m.

Fig. 2. Photomicrographs showing examples of $[^{35}\text{S}]$ single-labeled (A) and $[^{35}\text{S}]$ /DIG double-labeled neurons. Scale bar = 10 μm .

Fig. 3. Density ($\pm$ SEM) of GABA interneurons, identified by their content of GAD₆₇ mRNA, is decreased in schizophrenia and bipolar disorder. Asterisks denote significant difference at $p = 0.01$.

Fig. 4.

Density ($\pm$ SEM) of GABA interneurons that express GluR5 mRNA appears to be significantly decreased in schizophrenia and bipolar disorder, whereas density of those that express GluR6 mRNA is unaltered. Asterisks denote significant difference at $p = 0.01$.

Fig. 5. Density ($\pm$ SEM) of GABA interneurons that do not express GluR5 or GluR6 mRNA is decreased in schizophrenia and bipolar disorder. Asterisks denote significant difference at $p = 0.01$.

Fig. 6. Density ($\pm$ SEM) of neurons that do not express GAD₆₇ mRNA, which include all pyramidal neurons, but express the GluR5 mRNA is unchanged in schizophrenia or bipolar disorder.

Fig. 7.
The distribution of GABA cells that express different levels of GluR5 or GluR6 mRNA appears to be unaltered in schizophrenia or bipolar disorder.

Table 1

Cases used in the present study

Case	Age	PMI	Sex	Hemisphere	Race	CE	pH	Psychotropics received at time of death	Cause of death
<i>Bipolar disorder</i>									
1	83	17.5	M	R	W	0 (>12)	6.60	Divalproex	Cardiopulmonary failure
2	74	22.9	F	R	W	100	U	Divalproex, lorazepam, olanzapine	Cardiopulmonary failure
3*	26	22.8	F	L	W	0 (>62)	6.35	Lithium, lorazepam	Cardiopulmonary failure
4	52	17.2	F	R	W	200	U	Olanzapine	Hepatic failure
5	62	13.4	F	R	W	0 (>12.2)	U	Divalproex, bupropion, sertraline	Breast cancer
6	74	7.2	M	L	W	100	6.70	Gabapentin, olanzapine	Pneumonia
7	85	27.5	F	L	W	534	6.28	Olanzapine, divalproex	Cardiopulmonary failure
8*	51	31	M	L	W	0 (>23)	7.02	Clonazepam, gabapentin	Suicide by overdose
9	69	29.5	M	L	W	0 (>48)	U	Lithium	Pneumonia
10*	64	11	F	R	W	800	6.26	Trifluoperazine	Respiratory failure
11*	42	15.8	F	L	W	960	6.60	Lithium, divalproex, perphenazine	Medication overdose, rule out suicide
12	29	10.7	F	L	W	200	6.70	Divalproex, lithium, clonazepam, olanzapine	Cardiac arrest
13	36	9	M	L	W	U	U	Unknown	Suicide
14*	73	20.8	F	R	W	50	6.32	Carbamazepine, risperidone	Sepsis
15*	74	14.3	M	R	W	400	6.27	Divalproex, lithium, olanzapine, hydroxyzine prn, lorazepam prn, zolpidem prn	Pneumonia
16*	62	18.7	F	R	W	100	6.40	Divalproex, sertraline, risperidone, benzotropine, donepezil	Renal failure
17	82	5.0	M	L	W	U	6.37	Unknown	Cardiopulmonary failure
18	40	30.8	M	R	W	200	6.60	Gabapentin, ziprasidone, citalopram, risperidone, topiramate	Cardiac arrest
19	38	22	M	R	W	200	6.24	Divalproex, paroxetine, olanzapine	Carbon monoxide poisoning
20	47	16.3	F	R	W	50	U	Divalproex, topiramate, tiagabine, perphenazine, clonazepam,	Cardiopulmonary arrest
Mean	58.1±18.7	18.2±7.7	M:F=10:10	R:L=11:9		212.3±282.8	6.48±0.23		
<i>Schizophrenia</i>									
21	85	15.7	F	R	W	150	U	Risperidone, lorazepam	Sepsis
22	48	33.8	F	L	W	450	6.63	Risperidone, divalproex	Cardiac failure

Case	Age	PMI	Sex	Hemisphere	Race	CE	pH	Psychotropics received at time of death	Cause of death
23*	44	19	M	L	W	266	6.20	Clozapine	Pneumonia
24*	89	13.5	F	L	W	20	U	Trifluoperazine	Pneumonia
25	78	13.4	F	L	W	750	6.81	Haloperidol, lithium, cogenin	Sinus node disease
26	61	19.9	M	R	W	300	6.68	Clozapine	Sepsis
27	61	11	F	R	W	150	U	Paroxetine, clonazepam, clozapine	Myocardial infarction
28*	84	25.8	F	R	W	0 (71.6)	6.14	None	Cardiac arrest
29*	26	16	M	R	W	357	6.75	Fluphenazine decanoate	Suicide by hanging
30*	55	18	F	R	W	0 (6.7)	6.52	None	Oral cancer
<i>Schizophrenia</i>									
31*	47	19.2	M	R	W	0 (1.3)	6.57	Clonazepam, hydroxyzine prn	Lung cancer
32*	73	24	F	R	W	600	6.08	Risperidone, fluoxetine, clorazepate, midazolam prn	Lung cancer
33*	49	19	M	L	W	500	6.60	Haloperidol decanoate, lorazepam	Suicide by hanging
34	63	22.3	M	R	W	500	6.55	Clozapine, haloperidol, lorazepam, trazadone	Cardiac arrest
45	72	21.7	F	R	W	400	6.65	Risperidone, paroxetine	Ovarian cancer
36*	66	22.1	M	R	W	1000	6.43	Haloperidol	Emphysema
37*	83	23.2	F	R	W	2000	6.91	Haloperidol decanoate, fluphenazine decanoate	Gastrointestinal bleed
38	46	18.5	F	L	W	200	6.31	Olanzapine, divalproex	Sepsis
39	42	27.1	M	R	W	0 (1.5)	6.64	None	Leukemia
40	31	14	M	R	W	600	6.46	Risperidone, olanzapine, bupropion	Unknown (found dead in home)
Mean	60.2±18.5	19.8±5.4	M:F=10:10	R:L=14:6		412.2±465.7	6.53±0.23		
<i>Normal control</i>									
41	49	24.6	M	L	W		6.76	None	Myocardial infarction
42	37	18.8	M	R	W		6.68	None	Electrocution
43*	54	24.2	M	L	W		6.53	None	Cardiopulmonary arrest
44	78	14.1	F	R	W		6.22	None	Myocardial infarction
45	53	20.2	M	R	W		U	None	Cardiopulmonary arrest
46	65	24.3	F	R	W		6.40	None	Lung cancer
47	89	7.42	M	R	W		6.39	None	Cancer
48	69	15.3	M	R	W		6.88	None	Respiratory failure

Case	Age	PMI	Sex	Hemisphere	Race	CE	pH	Psychotropics received at time of death	Cause of death
49	74	12.5	F	L	W		6.33	None	Cardiopulmonary arrest
50*	66	7.4	F	R	W		6.03	None	Cancer
51	42	18.3	M	L	W		6.78	None	Myocardial infarction
52*	78	23.9	F	R	W		6.67	None	Breast cancer
53	40	16.6	M	L	W		6.24	None	Myocardiac infarction
54	67	22.3	M	L	W		6.42	None	Cardiopulmonary arrest
55*	70	22.5	F	L	W		6.26	None	Liver cancer
56*	66	18.7	M	R	W		6.76	None	Myocardial infarction
57	79	20.9	M	L	W		6.74	None	Cancer
58	38	28.8	M	L	W		6.53	None	Myocardiac infarction
59	30	14.8	M	R	W		U	None	Blunt force trauma
60	70	15	F	R	W		6.59	None	Cardiac arrest
Mean	60.7±16.7	18.5±5.7	M:F=13:7	R:L=11:9			6.51±0.24		

CE: Chlorpromazine equivalent in mg (numbers in parentheses denote medication-free duration in months); PMI: Post-mortem interval; U: Unavailable; Asterisks denote cases that have been included in a previous study (Woo et al., 2004).

Table 2Mean ($\pm$ S.D.) area (μm^2) of silver grain clusters

	Control	Schizophrenia	Bipolar Disorder
GluR5+/GAD67+	234.4 $\pm$ 55.6	232.0 $\pm$ 61.8	231.5 $\pm$ 56.9
GluR5+/GAD67-	240.5 $\pm$ 63.1	237.7 $\pm$ 61.0	242.2 $\pm$ 68.5
GluR6+/GAD67+	130.6 $\pm$ 51.0	127.3 $\pm$ 44.0	131.5 $\pm$ 56.9
GluR6+/GAD67-	131.1 $\pm$ 51.4	128.0 $\pm$ 51.7	139.1 $\pm$ 56.7